Dictyocaulus viviparus antigen for the diagnosis of lungworm infestation and for vaccination.

The invention relates to an antigen from the adult stages of the cattle lungworm Dictyocaulus viviparus (also termed D. viviparus or Dictyocaulus in that which follows) which can be used to detect lungworm infestation in cattle immunodiagnostically. In a vaccine, the antigen can induce immunoprotection against D. viviparus.

Lungworms are of great pathogenic and economic importance, particularly in small and large ruminants. Dictyocaulus is the only lungworm which achieves sexual maturity in cattle. It is found worldwide in places where moderate temperatures of 15-20°C predominate at least periodically. In Europe, D. viviparus is distributed endemically in the great river meadows, in rainy coastal regions and also on alpine pastures (R.J. Jörgensen (1980) Vet. Parasitol. 7, 153-167; H. Pfeiffer (1976) Wien. Tierärztl. Mschr. 63, 54-55). In the Netherlands, clinical dictyocaulosis was, for example, diagnosed in over 77% of the calf groups which were being maintained on pastures (J. Boch, R. Supperer (1992) Veterinärmedizinische Parasitologie [Veterinary Parasitology] 4th ed., Parey, Berlin, pp. 294-301).

In the calf which is exposed for the first time, the disease (dictyocaulosis) is caused by ingestion of the third larvae together with the pasture grass. By way of the blood system, the larvae reach the alveoli of the lungs, which they penetrate in order to reach the air-conducting parts of the lung. During this process, lesions are produced which serve as the port of entry for secondary bacterial infections; the multiplication of bacteria and other microbial pathogens leads to limited or generalized lung inflammations with all the possible sequelae such as pulmonary edema and heart failure (T. Schnieder, A. Bellmer, F.-J. Kaup (1989) Wien. Tierärztl. Mschr. 76: 372-476). Breathing is also substantially impeded by the adult stages, which are present in the upper airways and which lead to obstructions. Visible consequences of the

marked impairment of general well-being are reduced weight increases, or even weight losses, which are associated with growth delays. From time to time, the clinical symptoms worsen dramatically and rapidly lead to death.

Lungworm disease in cattle can be diagnosed on the basis of the clinical symptoms (G. Gräfner (1987) Monatsh. Vet. med. 42: 178-181) or on the basis of the larvae which are egested with the feces (J. Boch, R. Supperer (1992)). These possibilities are especially suitable for diagnosing the disease in the individual animal which is heavily infected. However, modern large-scale livestock farming requires epidemiological predictions and risk assessments with regard to the possibility of an outbreak of dictyocaulosis in the late pasture season, with these predictions and assessments being based on suitable diagnosis; i.e. many, possibly still only lightly infected calves have to be investigated in surveys using a safe and sensitive method. Serological methods are suitable for this purpose (A. Bellmer, T. Schnieder, A.M. Tenter (1989) Proc. 13th Conf. Wrld Ass. Adv. Vet. Parasit., pp. 33, Berlin, 07.-11.08.1989). Antigens which are identified in Dictyocaulus viviparus and then isolated, and in some cases prepared recombinantly, are used for the serodiagnosis. Medicaments which are active against adult and juvenile stages (e.g. Levamisol®, (pro) benzimidazoles, Netomin® or Ivermectin®) can be employed for treating the dictyocaulosis. These preparations are highly effective and are consequently usually able to prevent losses due to acute lungworm diseases (H. Mehlhorn, D. Düwel, W. Raether (1993) Diagnose und Therapie der Parasitosen von Haus-, Nutz- und Heimtieren [Diagnosis and Therapy of the Parasitoses of Domestic and Economically Useful Animals]. 2nd ed. Gustav Fischer Verlag, pp. 223-227). Because of their drastic effect, the active compounds may, in association with a prophylactic/metaphylactic treatment, possibly not allow the parasite to interact with the immune system of the host and consequently not allow a resilient (partial) immunity to develop and be maintained. The animals are then exposed, in an unprotected state, to an infection in the second year at pasture (COBS, D.E., S.R. Pitt, J. Förster, M.T. Fox (1987) Res. Vet. Sci. 43: 273-275).

For this reason, there have in recent times been evermore frequent demands, from the epidemiological point of view, for calves in the first season at pasture to be immunized, either by means of a low-grade subclinical infection or by means of vaccination. All that is currently available is a live vaccine in the form of X-ray-attenuated larvae which elicits a basal immunity which then has to be maintained by subsequent natural infection (Mehlhorn H., et al., (1993)). If subsequent immunization by way of natural infection is inadequate, breakthroughs, accompanied by coughing and disease, occur occasionally in association with sudden, heavy exposure to the parasite. Since the vaccine itself only has a shelf life of about 3 weeks in the refrigerator, it has to be stored carefully and used swiftly. This procedure forbids any "blanket" use; the vaccine therefore continues to be reserved in the main for special areas where the parasite is endemic. Because this vaccine lacks stability and quality, there is a need to develop defined vaccines (subunit vaccines). The object therefore arose of eliminating the cited disadvantages of the current vaccination method by preparing a novel, advantageous vaccine. This object was achieved by the present invention.

The invention relates to a novel, immunogenic native protein, termed DV 17, which was isolated from adult Dictyocaulus viviparus worms. Its immunogenicity is based, in particular, on the fact that after it has been administered subcutaneously to cattle, it induces an antibody response which confers immunoprotection on the animal. In addition, this protein can be used in an ELISA for retrospectively immunodiagnosing dictyocaulosis in cattle. DV 17 is characterized by the following physical properties. The protein is stable in all buffers employed. No decrease in immunoreactivity was observed after the purified antigen had been deep frozen (-85°C). Using an HPLC system and a Nucleosil C 18 column (150 mm x 4.6 mm; 5 u), the retention time for antigen DV 17 was measured to be 14 min (gradient elution comprising distilled water/0.1% TFA (= 0% B) and acetonitrile/0.1% TFA (= 100% B)). DV 17 has an estimated molecular weight of approx. 16,500 daltons in SDS-polyacrylamide gel electrophoresis (Phastgel 8-25%). The isoelectric point of DV 17 is in the range of 5.3-5.9. Finally, the part amino acid sequences depicted in Table 1 were determined following proteolysis with endopeptidase Lys C.

The ability to inhibit the development of Dictyocaulus viviparus in cattle following vaccination is an outstanding biological property of the protein.

The invention therefore relates to an immunogenic protein having a protective effect, which protein is isolated from adult worms of the lungworm Dictyocaulus viviparus and preferably has a molecular weight of 15,000-18,000 Da, an isoelectric point between 5.3 and 5.9 and amino acid part sequences as depicted in Table 1.

The invention preferably relates to a protein which has a molecular weight of 16,500 ± 1500 Da and/or an isoelectric point of 5.6.

The invention furthermore relates to a protein which comprises the amino acid sequence depicted in Table 6 (SEQ ID NO.: 30) or parts thereof.

The invention furthermore relates to a process for isolating a protein which comprises using extraction methods and chromatographic methods which are known to the skilled person to carry out the isolation.

The invention furthermore relates to a DNA which encodes a protein as described above, preferably to a DNA which comprises a DNA sequence depicted in Table 1.

The invention furthermore relates to a DNA which

(a) comprises a DNA sequence depicted in Table 6 (SEQ ID NO.: 29) or parts thereof, or

(b) hybridizes, under stringent conditions, with a DNA sequence according to (a).

The invention also relates to a process for isolating said DNA, which comprises

a) preparing degenerate oligonucleotides which comprise a DNA sequence depicted in Table 1, or parts thereof,

b) labeling the oligonucleotides which have been prepared according to a) radioactively or non-radioactively, and

c) isolating cDNA clones from a cDNA library prepared from Dictyocaulus viviparus, which cDNA clones hybridize, under stringent conditions, with the hybridization probes which have been prepared in accordance with b).

The invention also relates to a process for isolating said DNA, which comprises

a) preparing PCR primers which comprise a DNA sequence depicted in Table 1, or parts thereof, or which comprise an oligo-dT sequence,

b) using the resulting PCR primers to generate PCR fragments from a cDNA library prepared from Dictyocaulus viviparus,

c) cloning and analyzing these fragments in accordance with current methods, and

d) using these fragments in place of the degenerate oligonucleotides to complete the cDNA sequence by means of hybridization methods as described above.

The latter process can also be modified such that RNA is used as the template for the PCR reaction, with this RNA initially being reverse-transcribed in an additional step and the resulting first strand of cDNA being used for the PCR.

The invention furthermore relates to a recombinant protein which comprises amino acid part sequences depicted in Table 1 and which can preferably be obtained by expressing one of the cDNAs obtained as described above in prokaryotes or eukaryotes and then purifying this expressed protein using methods which are known to the skilled person.

The invention likewise relates to an immunochemical process which uses the above-described D. viviparus protein to determine the quantity of DV 17-specific antibodies in the blood of cattle, which comprises incubating DV 17-coated ELISA plates with

the cattle serum to be investigated and detecting any DV 17/antibody complexes formed using peroxidase-conjugates, polyclonal antibodies and an appropriate color reaction known to the skilled person.

The invention also relates to the use of the above-described D. viviparus protein as a vaccine, in association with a carrier or adjuvant and, where appropriate, auxiliary substances, for immunizing cattle against dictyocaulosis.

The invention also relates to a diagnostic kit which comprises the above-described D. viviparus protein.

Finally, the invention relates to a vaccine which comprises the above-described D. viviparus protein and a carrier, an adjuvant and, where appropriate auxiliary substances.

The invention will now be clarified in detail with the aid of examples without being restricted to these examples. The tables are described as follows:

Table 1: Part amino acid sequences of the isolated and concentrated Dictyocaulus viviparus DV 17 protein, and the degenerate nucleotide sequence which can be deduced from these sequences. Abbreviations: N = A, G, C, T; Y = T, C; H = A, C, T; R = A, G; M = A, C

Table 2: Degenerate primers used for amplifying DV 17 DNA. Abbreviations, see description to Table 1

Table 3: PCR fragments which have been obtained from DV RACE cDNA using the degenerate primers from Table 2

Table 4: Gene-specific primers from RACE experiments for producing DV 17 cDNA in which the molecule has a complete 3' end

Table 5: DV 17 cDNA fragments obtained by means of RACE

Table 6: cDNA and protein sequences of DV 17.

Normal cattle sera and cattle sera from animals which were infected with gastrointestinal nematodes such as Ostertagia ostertagi and Cooperia oncophora and also the lungworm Dictyocaulus viviparus were used for identifying protein DV 17.

Chromatographically separated protein fractions obtained from homogenized adult lungworms were subjected to further fractionation by means of SDS polyacrylamide gel electrophoresis and immobilized on Immobilon P membranes (semidry blotting). The lungworm-specific protein DV 17 was then detected using the specific sera from infected animals and then purified further using a reverse phase HPLC column. The purity of the protein fraction was checked in silver-stained SDS polyacrylamide gels (Phastgels). A BCA protein assay was used to determine the protein concentration in electrophoretically pure DV 17 fractions and the latter were then deep frozen at -85°C. Helminth-naive cattle were vaccinated 2 times with a defined quantity of purified DV 17 on each occasion. One week after the second vaccination, the cattle were challenged with Dictyocaulus viviparus L3 larvae. Unvaccinated animals served as controls. 4 weeks after the challenge, the cattle were slaughtered and the number of adult worms in the lung was determined and the lengths of the male and female worms were measured. The reduction in the number of adult worms as compared with the unvaccinated control was defined as the measure of the immunoprotection.

In connection with DNA hybridization, "stringent conditions" denotes 6xSSC, 68°C, in the present application.

The PCR conditions have to be determined in preliminary experiments using methods which are known to any skilled person.

Example 1: Preparation of sera from infected animals

6-month-old helminth-naive cattle were infected with different doses of third larvae from various nematode species (Dictyocaulus viviparus, Ostertagia ostertagi and Cooperia oncophora). In the Dictyocaulus group, the infection doses were 2500, 1250 and 500 larvae/animal; 3 animals were used at each dose. Infection doses of 70,000, 30,000 and 15,000 larvae/animal were chosen for the Ostertagia group. As well as a non-infected group (= negative control group), a mixed group was also included in which each animal was infected with 2500 Dictyocaulus larvae, 10,000 Ostertagia larvae and 10,000 Cooperia larvae. Serum samples, which were aliquoted and stored at -25°C, were taken from each animal on days D0 (= day of infection), D +21, D +40, D +56, D +70, D +84, D +98 and D +112. The sera were used to identify the Dictyocaulus DV 17 antigen in electrophoretically and chromatographically separated protein fractions and also for assessing specificity.

Example 2: Isolation of adult lungworms

6-month-old, helminth-naive cattle were infected orally with in each case 5000 Dictyocaulus viviparus third larvae; the animals were then given the same infection dose on the following day. 28 days after the infection, the cattle were slaughtered and adult worms were collected from the lungs after dissection. The worms were then washed 3x with phosphate-buffered sodium chloride solution, weighed and stored at -85°C until worked up.

Example 3: Extraction of DV 17 from adult lungworms

10 g of frozen worm mass were thawed at room temperature and then homogenized together with 40 ml of a 0.025 M solution of Tris-HCl, pH 7.4, containing 2 mM Pefabloc®, in a tissue homogenizer. In order to remove coarse tissue constituents, the homogenate was centrifuged at 3010 g for 15 min at 4°C and the pellet was

discarded. The supernatant was centrifuged at 39,800 g for 20 min at 4°C and the supernatant from this centrifugation was then recentrifuged under the same conditions for 10 min. Following filtration using 1.2 µm filters, the clear supernatant was dialyzed (cut-off of the dialysis membrane, 8000) at 4°C overnight in 1 liter of phosphate-buffered sodium chloride solution (PBS).

Example 4: Preparative gel filtration

The dialyzed supernatant was centrifuged at 39,800 g for 15 min and the clear supernatant was fractionated in a Pharmacia FPLC system using a preparative gel filtration column (column type XK 16/60; separation medium: Superdex 75 prep grade, column volume: 124 ml). PBS, pH 7.4, was used for the elution. The fractions having the retention volumes 65-75 ml were collected and concentrated using ultrafiltration modules (cut-off 3000). Protein DV 17 was detected using an amplified Western blot.

Example 5: Western blot analysis

The concentrated Superdex 75 prep grade fraction was mixed with reduced SDS buffer in a ratio of 1:2; 40 µl of this mixture were added to each well on an SDS-Excel gel (from Pharmacia). The electrophoresis was carried out in a Multiphor II chamber (from Pharmacia) under standardized running conditions (600 V, 50 mA, 30 W, running time: 90 min). The electrophoretically separated proteins were transfered onto Immobilon P membranes by means of semidry blotting (Tovey ER, Baldo BA. Electrophoresis. 8, 1987, 384-387) (transfer conditions: 45 min, constant current strength of 0.8 mA/cm ^{2}) and, after a 24-hour blocking phase using 3% bovine serum albumin in Tris-buffered sodium chloride solution (TBS), incubated for 1 hour with a Dictyocaulus-specific immune serum (obtained on D+40, see Example 1) used at a dilution of 1:20. Normal bovine serum (dilution 1:20) was used as the negative control. After having been washed 3 times with TBS + 0.05% Tween 20,

the blot membrane was incubated for 1 hour with a biotin-labeled goat anti-bovine IgG (H+L) antibody (1:500; from Pierce). After having been washed (TBS + 0.05% Tween 20) 3 times, the membrane was incubated for 1 hour with a biotin/streptavidin/alkaline phosphatase enzyme conjugate (1:2500; from Pierce). The substrate was developed using the substrate kit supplied by Biorad.

Example 6: Reverse phase HPLC

After the DV 17 had been immunologically identified in the preparative gel filtration fractions, the protein was purified further by means of HPLC. A Nucleosil C 18 5U column from Alltech (150 mm x 4.6 mm) was used for this purpose. The protein was eluted with a linear buffer gradient (buffer A: high-purity water +0.1% trifluoroacetic acid (TFA); buffer B: acetonitrile + 0.1% TFA). 500 µl of the fraction which was concentrated in Example 4 were diluted 1:2 with buffer A and then injected into the column. The flow rate was 0.5 ml/min. The gradient elution was started 5 min after the injection and terminated 10 min later. DV 17 was found to have a retention time of 14 min.

Example 7: Demonstration of purity and determination of molecular weight

The HPLC-purified fraction having a retention time of 14 min was analyzed on a Phast system (Pharmacia) SDS polyacrylamide gel (8-25% Phast SDS gel) under standardized conditions. The "Silver Stain SDS-PAGE Standards, low range" kit supplied by Biorad was used for the molecular weight markers. After the electrophoresis, DV 17 was visualized by silver staining (Pharmacia Silverstain kit). The molecular weight was determined with a videodensitometer (Biorad Molecular Analyst) using the "Profile analyst II" evaluation program. DV 17 was calculated to have a molecular weight of 16,500 ± 1500 daltons.

Example 8: Determination of the isoelectric point

DV 17 which had been isolated as described in Example 6 was diluted with high-purity water and loaded onto previously prepared focusing gels (pH 3-10 IEF Phast gels, from Pharmacia). The focusing in the Phast system was carried out under standardized conditions. Marker proteins having defined isoelectric points (pH 3.5-9.3, from Pharmacia) were included for the purpose of determining the isoelectric point of DV 17. The latter was found to have an isoelectric point of 5.3-5.9.

Example 9: Amino acid sequence analysis

DV 17 which had been isolated and concentrated as described in Example 6 (40 µg) was cleaved with the endopeptidase Lys C and the resulting peptides were purified on a C 18 reverse phase HPLC column. The N termini of 7 peptides were sequenced. The 7 part amino acid sequences shown in Table 1 were identified.

Example 10: Demonstration that DV 17 has a protective effect

5-month-old helminth-naive bull calves (stall-reared, negative by coproscopic detection) were in each case vaccinated subcutaneously with 50 µg of purified, native DV 17. 4 weeks later, the calves were given a booster vaccination of 45 µg of antigen/animal. 1 week after the 2nd vaccination, the animals were in each case challenged with 2 x 1000 Dictyocaulus viviparus L3 larvae. Unvaccinated animals were used as controls. 35 days after the challenge, the animals were slaughtered and the number of adult worms in the lung was determined; at the same time, the lengths of intact male and female worms were measured. The number of adult worms was found to be reduced by 80% in the vaccinated group. Adult worms from the vaccinated group were significantly smaller than those from the control group (reduction of 33%).

Example 11: ELISA for the serological detection of dictyocaulosis

ELISA plates (Maxisorb from Nunc) were coated with a concentration of 5 µg of DV 17/ml of PBS; the reaction volume was 100 µl per well. After having been incubated at 37°C for 1 hour, the plates were washed 3 times in an ELISA washer, with the wash volume being 200 µl per well. The washing solution used was high-purity water +0.1% Tween 20. Nonspecific binding sites were blocked by incubating (3 hours at room temperature) with a proteolytic mixture of gelatin (from Boehringer Mannheim). The incubation took place on a microtiter plate shaker at a shaking frequency of 300 rpm. After the plates had been washed 3 times, the wells were loaded with a 1:200 dilution of specific infection sera and control sera and incubated at room temperature for 1 hour. After the plates had been washed 3 times, peroxidase-conjugated, polyclonal rabbit antibody against bovine IgG (Fc fragment-specific; from Dianova) was used, at a dilution of 1:10,000, as the detection antibody; the duration of the incubation was 30 min. The plates were then washed 4 times and incubated with substrate solution (composition: 5 ml of 10 times concentrated ABTS buffer + 45 ml of high-purity water + 1 tablet of ABTS (^ 50 mg of ABTS). Substrate development took place at room temperature and was monitored every 10 min in an ELISA reader at 414 nm. In the ELISA, it was possible to detect Dictyocaulus-specific antibodies 20 days after a lungworm infection at the earliest.

Example 12: Cloning and sequencing of the DV 17 antigen

"DYNABEADS" (DYNABEADS mRNAdirect hit, DYNAL) were used to isolate total D. viviparus RNA. 400 ng of this RNA was precipitated with ethanol and used as the template for a RACE (reamplification of cDNA ends; Frohmann et al., 1988, Proc. Natl. Acad.Sci. U.S.A. 85:8998-9002) cDNA synthesis, employing the "Marathon cDNA Amplification Kit" (Clontech). The cDNA adapter-specific primers from the said kit, and the "Expand Long Template PCR System" (Boehringer Mannheim) were used. Conditions: 600 nM of each primer; 1.75 mM $MgCl_2$, 400 mM dNTPs, 392 ng

of Taq Start Antibody (Clontech) and 0.35 U of DNA polymerase mix. The temperature profile was as follows:

Cycle 1	92°C	120 s
	60°C	60 s
	68°C	360 s
Cycles 2-30	92°C	60 s
	60°C	60 s
	68°C	360 s

The amplified DV 17 RACE cDNA was diluted 1:20 in tris-glycine buffer (from the Marathon cDNA Amplification Kit) for use as the template in PCR reactions.

The RACE cDNA was used to amplify a region of the DV 17 cDNA sequence by means of PCR, employing degenerate primers whose sequences were obtained from the peptide sequences of the purified antigen. In this amplification, the primers were provided with restriction enzyme cleavage sites which then facilitated the subsequent subcloning of the resulting PCR fragments. The primers employed are listed in Table 2.

Five DNA fragments were obtained after 2 rounds of PCR in a PE 9600 Thermal Cycler (Perkin Elmer) (500 nM of each primer; 0.1 vol of PCR buffer; 200 mM dNTPs; 1.25 units of Ampli Taq Gold (Perkin Elmer). The following temperature profile was used:

Cycle 1	92°C	600 s
Cycles 2-31	94°C	40 s
	55°C	40 s
	72°C	60 s

Four of the fragments (see Table 3) were obtained in sufficiently large yields to be gel-purified and sequenced with the aid of the primers which were used for the preparation.

Using the "Geneworks" (IntelliGenetics) program to align the resulting sequence data resulted in two contigs. Taken together, these two contigs contained 5 or 6 unambiguous peptide sequences which were obtained from the native antigen. Non-degenerate (gene-specific) primers (see Table 4), which were obtained from these sequence data, were used in RACE experiments, together with the Marathon cDNA adapter-specific primers in order to obtain DNA fragments which constitute the 3' end of the cDNA molecule.

The RACE reaction mixtures contained 600 nM concentrations of each primer; 0.2 µl of diluted, amplified DV 17 RACE cDNA; 0.1 vol of PCR buffer I; 200 µM dNTPs and 1.25 U of Ampli Taq Gold. The temperature profile was the same as in the PCR reaction using the non-degenerate primers apart from the fact that the annealing temperature was 60°C and the samples were left for 360 s at 72°C in the last cycle.

Two PCR fragments were obtained using the gene-specific primers (Table 5). After the RACE, the fragments were gel-purified, subcloned and sequenced using a T7 promoter sequencing primer (Promega; sequence: TAATACGACTCACTATAGGG, SEQ ID NO.: 31).

An alignment was carried out (Geneworks) using the sequences of these fragments and those of the fragments which were obtained with the non-degenerate primers and is depicted in Table 6.

Table 1

	1				5			
1.	Ser	Glu	Ser	Leu	Tyr	Glu	Lys	(SEQ ID NO.: 1)
	TCN	GAR	UCN	YTN	TAY	GAR	AAR	(SEQ ID NO.: 8)

	1				5					
2.	Met	Met	Asp	Asn	Phe	Val	Lys			(SEQ ID NO.: 2)
	ATG	ATG	GAY	AAY	TTY	GTN	AAR			(SEQ ID NO.: 9)
	1				5					10
3.	Tyr	Lys	Asp	Glu	Asn	Glu	Phe	Met	Asp	Ala
	TAY	AAR	GAY	GAR	AAY	GAR	TTY	ATG	GAY	GCN
				14						
	Leu	Lys	Gln	Lys						(SEQ ID NO.: 3)
	YTN	AAR	CAR	AAR						(SEQ ID NO.: 10)
	1				5					10
4.	Tyr	Asp	Ile	Pro	Glu	Gln	Tyr	Arg	Glu	Ile
	TAY	GAY	ATH	CCN	GAR	CAR	TAY	MGN	GAR	ATH
					15					20
	Ile	Pro	Gln	Asn	Val	Ala	Glu	His	Leu	Lys
	(SEQ ID NO.: 4)									
	ATH	CCN	CAR	AAY	GTN	GCN	GAR	CAY	YTN	AAR
	(SEQ ID NO.: 11)									
	1				5					10
5.	Asp	Ala	Ile	Glu	Lys	Tyr	Glu	Asp	Ile	Pro
	GAY	GCN	ATH	GAR	AAR	TAY	GAR	GAY	ATH	CCN
					15					20
	Glu	Gln	Tyr	Arg	Glu	Ile	Ile	Pro	Gln	Asn
	GAR	CAR	TAY	MGN	GAR	ATH	ATH	CCN	CAR	AAY

	Val	Ala	Glu	His	Leu	Lys				(SEQ ID NO. 5)
	GTN	GCN	GAR	CAY	YTN	AAR				(SEQ ID NO.: 12)
	1				5					10
6.	Phe	His	Ala	Glu	Leu	Leu	Ala	Gly	Ile	Lys
	TTY	CAY	GCN	GAR	YTN	YTN	GCN	GGN	ATH	AAR
					15					
	Pro	Ser	Leu	Glu	Glu	Leu	Lys	Lys		(SEQ ID NO.: 6)
	CCN	TCN	YTN	GAR	GAR	YTN	AAR	AAR		(SEQ ID NO.: 13)
	1				5					10
7.	Gln	Phe	Pro	Ile	Leu	Thr	Ser	Val	Phe	Ser
	CAR	TTY	CCN	ATH	YTN	ACN	TCN	GTN	TTY	TCN
				14						(SEQ ID NO.: 7)
	Asn	Glu	Glu	Lys						(SEQ ID NO.: 14)
	AAY	GAR	GAR	AAR						

Table 2

Oligo	Orientation	DNA/amino acid sequence
A055/1001	sense	CCG GAA TTC GAY GCN ATN GAR AAR TAY GA
		(SEQ ID NO.: 15)
		EcoRI D A I E K Y E
		(SEQ ID NO.: 16)
A055/1002	sense	CGC GGA TCC GAR ATN ATN CCN CAR AAY GT
		(SEQ ID NO.: 17)
		BamHI E I I P Q N V
		(SEQ ID NO.: 18)
A055/1003	sense	CCG GAA TTC TAY AAR GAY GAR AAY GAR TT
		(SEQ ID NO.: 19)

EcoRI Y K D E N E F

(SEQ ID NO.: 20)

A055/1004 antisense AAAA CTG CAG NGC RTC CAT RAA YTC RTT YTC

(SEQ ID NO.: 21)

PstI A D M F E N E

(SEQ ID NO.: 22)

A055/1005 antisense AAAA CTG CAG YTT YTC YTC RTT RCT RAA NAC

(SEQ ID NO.: 23)

PstI K E E N S F V

(SEQ ID NO.: 24)

Table 3

Primer combination	Approximate fragment size (bp)*	Sequenced
A055/1001:A055/1004	180	Yes
A055/1001:A055/1005	480	No
A055/1002:A055/1004	150	Yes
A055/1002:A055/1005	470	Yes
A055/1003:A055/1005	400	Yes

* determined by gel electrophoresis

Table 4

A075/1001 GAC ATG CAT GTA GAC GCA CTT GGA GAA GAG GC

(SEQ ID NO.: 25)

NsiI V D A L G E E A

(SEQ ID NO.: 26)

A108/1001 CCG GAA TTC CCT GAA CAG TAC AGA GAG ATC ATT CCA

(SEQ ID NO.: 27)

EcoRI P E Q Y R E I I P

(SEQI ID NO.: 28)

Table 5

Primer combination*	Fragment size (bp)	Cloning vector (Promega)
A075/1001:AP1	342	pGEM7Zf-
A108/1001:AP1	541	pGEM3Z

*AP1 is the Marathon cDNA adapter-specific primer

Table 6

TGGAGAARTA CGAAGATATT CCTGAACAGT ACAGAGAGAT CATTCCACAA

E K Y E D I P E Q Y R E I I P Q

AACGTGGCCG AGCATTTAAA ATCGATAACT GAGGAAGAGA AAAAAGTGCT

N V A E H L K S I T E E E K K V L

CAAAGAATTT GTTAAAGACT ATGCAAAATA CAAAGATGAA AATGAGTTCA

K E F V K D Y A K Y K D E N E F M

TGGACGCATT AAAGCAAAAA TCTGAAAGCC TTTATGAGAA AGCTAAAAAA

D A L K Q K S E S L Y E K A K K

CTTCAAGATT TGCTGAAATC AAAAGTAGAC GCACTTGGAG AAGAGGCAAA

L Q D L L K S K V D A L G E E A K

ACAATTTGTG ATGAAGCTTA TCGCTGAGGC TCGTAAATTC CACGCAGAGC

Q F V M K L I A E A R K F H A E L

TACTGGCCGG CATCAAACCA TCGCTAGAAG AACTAAAAGC CGTCGCTAAA

L A G I K P S L E E L K A V A K

AAGCATATTG AAGAGTTTGA GAAGTTATCA GATGCAGCTA AAGATGATTT

K H I E E F E K L S D A A K D D F

CAAAAAGCAA TTCCCTATCC TCACATCCGT GTTCAGCAAT GAAAAAGCAA

K K Q F P I L T S V F S N E K A K

AGAAAATGAT GGACAACTTT GTGAAAAATT AAAGTTGTAT GATTTGCAGG

K M M D N F V K N (SEQ ID NO.: 30)

ATATGAAATA AATGTTAAAT TGAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AG (SEQ ID NO.: 29)